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RESEARCH PAPER

Brassinosteroid biosynthesis and signalling in *Petunia hybrida*

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Abstract

Brassinosteroids (BRs) are steroidal plant hormones that play an important role in the growth and development of plants. The biosynthesis of sterols and BRs as well as the signalling cascade they induce in plants have been elucidated largely through metabolic studies and the analysis of mutants in *Arabidopsis* and rice. Only fragmentary details about BR signalling in other plant species are known. Here a forward genetics strategy was used in *Petunia hybrida*, by which 19 families with phenotypic alterations typical for BR deficiency mutants were identified. In all mutants, the endogenous BR levels were severely reduced. In seven families, the tagged genes were revealed as the petunia BR biosynthesis genes *CYP90A1* and *CYP85A1* and the BR receptor gene *BRI1*. In addition, several homologues of key regulators of the BR signalling pathway were cloned from petunia based on homology with their *Arabidopsis* counterparts, including the BRI1 receptor, a member of the BES1/BZR1 transcription factor family (PhBEH2), and two GSK3-like kinases (PSK8 and PSK9). PhBEH2 was shown to interact with PSK8 and 14-3-3 proteins in yeast, revealing similar interactions to those during BR signalling in *Arabidopsis*. Interestingly, PhBEH2 also interacted with proteins implicated in other signalling pathways. This suggests that PhBEH2 might function as an important hub in the cross-talk between diverse signalling pathways.

Key words: BES1, BIN2, brassinosteroids, BRI1, BZR1, *compact disc*, dwarf, *Petunia hybrida*, SEC.

Introduction

The importance of steroidal hormones in mammalian development has already been known for almost eight decades. The idea that plant steroids have hormonal functions arose when scientists reported the isolation of brassinolide (BL), a steroid with strong plant growth-promoting activity, from bee-collected pollen of *Brassica napus* L. (Grove et al., 1979). Brassinosteroids (BRs) are widely distributed among the plant kingdom and so far they have been found in gymnosperms, monocots, dicots, a pteridophyte, a bryophyte, and a chlorophyte (Bajguz and Tretyn, 2003). The subsequent identification of several BR biosynthesis and signalling mutants further supported the importance of these compounds for growth and development, and nowadays BRs are accepted as phytohormones. BRs are involved in various growth and

developmental processes such as cell division and elongation, vascular differentiation, abiotic and biotic stresses, photomorphogenesis, germination, senescence, and fertility (Clouse and Sasse, 1998; Bajguz and Hayat, 2009).

The sterol biosynthesis pathway can be divided into three domains (Fig. 1). In the first part, the mevalonate-derived squalene is converted via multiple steps into 24-methylene lophenol. Subsequently, the pathway diverges into two downstream branches. One branch produces sitosterol and stigmasterol, while the other produces campesterol. These sterols are integrated into cellular membrane components. In addition, the biosynthetic precursor campesterol is utilized to synthesize BRs (Lindsey et al., 2003). Plants have multiple ways to synthesize BL from campesterol. Detailed studies on the BR biosynthesis route in *Catharanthus roseus* and *Arabidopsis thaliana* led to the identification of two parallel branched routes, better known as the early and late C-6 oxidation pathways that converge at catasterone (Fig. 1). The two pathways are linked to each other, implying that they are not totally autonomous (Shimada et al., 2001). Although the early and late C-6 oxidation pathways are found throughout the plant kingdom, it seems that in species such as tomato and tobacco the late C-6 oxidation pathway is more prevalent (Nomura et al., 2001). Fujioka et al. (2002) identified an early C-22 oxidation pathway, also named the CN-independent pathway, that merges into the late C-6 oxidation pathway, constituting the major route to produce biologically active BRs as shown in Fig. 1 (Onishi *et al*., 2012; Zhao and Li, 2012). In addition, two shortcuts were reported that convert 22-OH-3-one and 3-epi-6-deoxoCT via C-23 hydroxylation to 6-deoxy3DT and 6-deoxyTY, respectively (Ohnishi et al., 2006).

A number of sterol and BR biosynthesis mutants have been described, predominantly in *Arabidopsis*, and many of the genes have been cloned and studied (reviewed by Clouse, 2002). BR biosynthesis mutants and some sterol mutants such as *dwarf7* (*dwf7*), *diminuto* (*dim*), and *dwarf5* (*dwf5*) can be rescued by exogenous application of BR. However, mutants that are affected in genes functioning in early steps of the sterol biosynthesis pathway such as *FACKEL* (*FK*), *STEROL METHYL TRANSFERASE 1* and *2* (*SMT1*, *SMT2*), and *HYDRA1* (*HYD1*) are embryo lethal and cannot be rescued by application of exogenous BR. Presumably, the absence of these sterols interferes with membrane integrity.

BR signalling has primarily been elaborated by studies in *Arabidopsis*. The currently known BR signalling pathway in *Arabidopsis* runs from membrane to nucleus (reviewed by Clouse, 2011). A key event is the degradation of a negative regulator of BR signalling, the *Arabidopsis* GSK3/SHAGGY-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2; Kim et al., 2009). The negative effect of BIN2 on BR signalling is performed via phosphorylation of the closely related plant-specific transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINOZOLE RESISTANT 1 (BZR1; Wang et al., 2002; Yin et al., 2002). It was shown that other *Arabidopsis* GSK3-like protein kinases (ASKs) act redundantly with BIN2 in the BR signalling pathway (Vert and Chory, 2006; Kim et al., 2009; Yan et al., 2009; Rozhon et al., 2010). Phosphorylation of BES1/BZR1 results in inhibition of their activity through proteasome-mediated degradation, cytoplasmic retention by 14-3-3 proteins, and inhibition of their DNA binding and transcription activation activities (Li et al., 2001; He et al., 2002; Yin et al., 2002; Vert and Chory, 2006; Gampala et al., 2007; Ryu et al., 2007).

Here an extensive analysis of BR biosynthesis and signalling in petunia is presented. The goal of the study is to examine to what extent the biosynthesis of steroids and BRs and the signalling cascades downstream in plants are conserved. The authors' petunia collection was browsed for mutants that exhibit a typical BR deficiency phenotype; five BR biosynthesis or signalling mutants were identified and their BR content was determined. In three cases, the tagged genes could be identified. Furthermore, several petunia homologues of key components of the BR signalling pathway in *Arabidopsis* were isolated and it was determined if a similar network of interactions between these factors can occur. It was also found that several components of other hormone pathways could interact with the petunia homologue of a member of the BES1/BZR1 family of transcription factors. These interactions reveal new mechanisms, not previously recognized, that suggest extensive cross-talk between BR signalling and other hormone signalling pathways.

Materials and methods

Brassinolide application

Young plantlets were each sprayed each 2 d or 3 d until run-off with 1 µM 24-epibrassinolide (Wako Biochemistry) or with a solvent control (0.01% ethanol and 0.1% Tween-20) for a couple of weeks.

Purification and GC-MS analysis of sterols and BRs in petunia tissue

Petunia plantlets were freeze-dried and subjected to extraction and chromatography according to Nomura et al. (1999). In high-performance liquid chromatography, fractions 48/49 (22-OH-CR and 28-nor-6-deoxoCT), 50/51 (22-OH-3-one, 6-deoxoCT), and 52/53 (3-epi-6-deoxoCT) were collected in addition to the fractions cited in Nomura et al. (1999). The respective BRs were derivatized and analysed by gas chromatography–mass spectrometry (GC-MS) as described (Nomura et al., 2001) except that a Shimadzu GC-MS2010 was used. Sterols in petunia were analysed according to Nomura et al. (2004).

Genomic DNA isolation and PCR

Petunia DNA was isolated by grinding one young leaf in liquid nitrogen. To this 400 µl of DNA extraction buffer was added (0.1 M TRIS pH 8.0, 0.5 M NaCl, 50 mM EDTA, and 10 mM β-mercaptoethanol) and samples were incubated for 15–30 min at 65 °C. Next 250 µl of phenol/chloroform was added, and samples were shaken and subsequently centrifuged for 5 min at maximum speed in a microcentrifuge. The supernatant was transferred to a clean tube and DNA was precipitated using 2-propanol and washed with 70% ethanol. The pellet was dissolved in 100 µl of H_2O containing DNase-free RNase (10 mg ml^{-1}) followed by an incubation step for 15–30 min at 65 °C. PCRs were performed by using 2 µl of genomic DNA in a standard PCR. The nucleotide sequences of the primers used are listed in Supplementary Table S1 available at *JXB* online.

Isolation of 3' gene fragments by rapid amplification of cDNA ends (RACE) PCR

In order to isolate cDNA fragments, total RNA was isolated from leaves followed by first-strand cDNA synthesis using the RACE1 primer as described previously (Quattrocchio et al., 2006). PCR

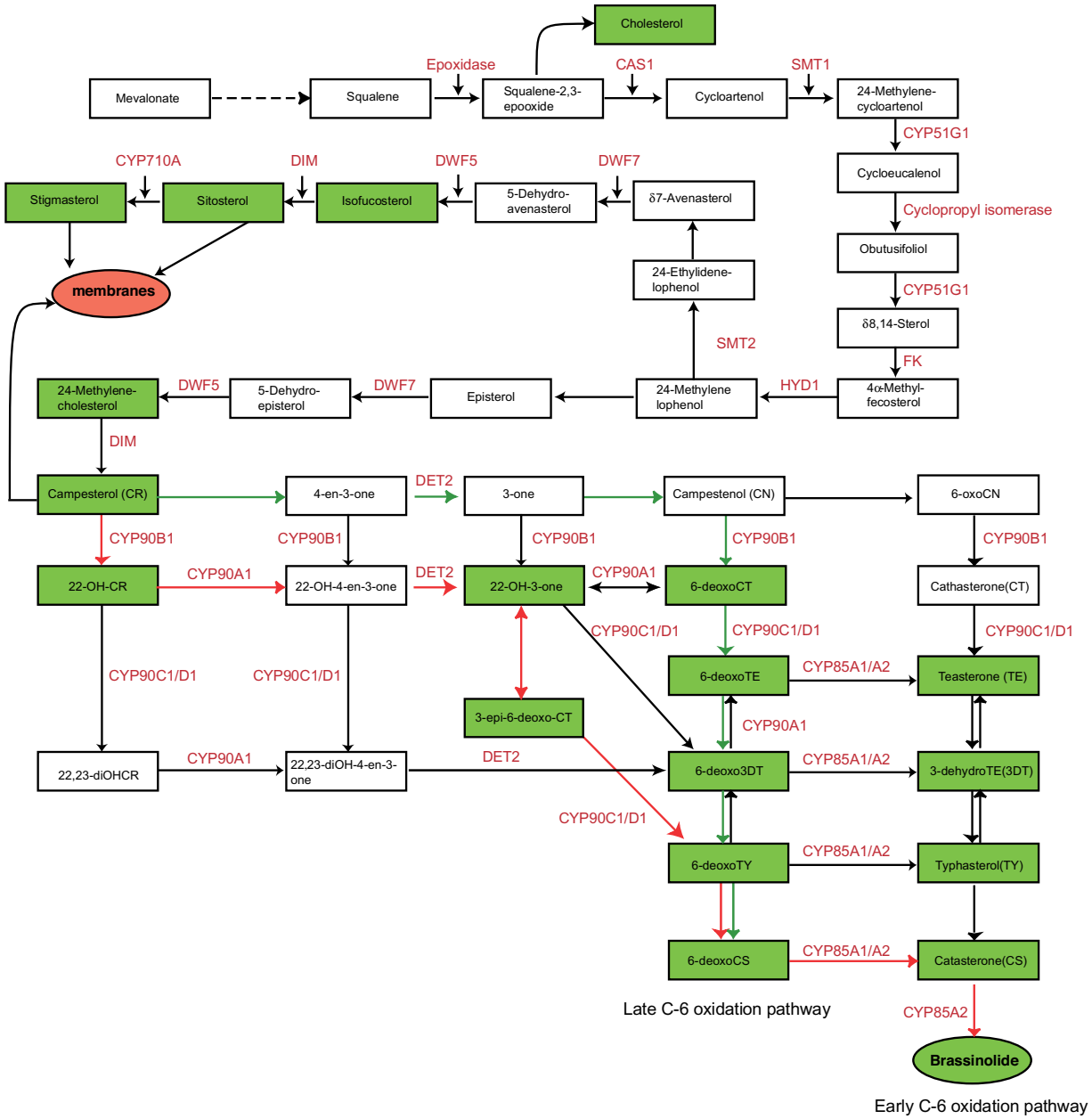


Fig. 1. Biosynthesis of steroids and BRs, and the *Arabidopsis* enzymes involved. The metabolic products are shown in boxes and the enzymes involved in the pathway are depicted in red. The predominant pathway to synthesize BL runs from the campestanol (CN)-independent pathway and late C-6 oxodation pathway and is indicated by the red arrows. The CN-dependent pathway is represented by the green arrows. Compounds measured in this study are indicated in a green box.

amplification was carried out using gene-specific primers or, to isolate 3' ends of cDNAs, by using one gene-specific primer in combination with the RACE2 primer under standard PCR conditions (annealing temperature 55 °C). All primer sequences used are listed in Supplementary Table S1 at *JXB* online. PCR fragments were directly sequenced or cloned in pGEM-Teasy (Promega) and sequenced.

Isolation of 5' gene fragments by SOTI-PCR

To complete sequences at the 5' end, for some genes a somatic insertion-mediated PCR was performed (Rebocho et al., 2008). Here, somatic *dTph1* insertions in the target gene that may occur in some cells of petunia W138 plants were used. W138 genomic DNA was subjected to nested PCR using gene-specific primers and *dTph1* transposon primers (see Supplementary Table S1 at *JXB* online). In the first PCR, the *dTph1* transposon primers out11 or out12 were used in a standard PCR with annealing temperature 55 °C. In the second, nested PCR, 1 µl of the out11 reaction was re-amplified with out15 and a nested gene-specific primer, while the out12 reaction was re-amplified with out6 and a nested gene-specific primer. The second reaction was a touch-down PCR where annealing started at 70 °C and decreased by 1 °C every cycle until reaching 55 °C, followed by 25 additional cycles at 55 °C. PCR fragments were directly sequenced or cloned in pGEM-Teasy (Promega) and sequenced.

Phylogenetic analysis

Phylogenetic trees were generated using the Neighbor–Joining method of ClustalX (ftp://ftp.ebi.ac.uk/pub/software/clustalw2/). Bootstrap mode (1000 replications) was used for estimating the level

of confidence assigned to the particular nodes in the tree. The tree was visualized with the help of Treeviewer 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and rooted using ClustalW (http://www.bioinformatics.nl/tools/clustalw.html). Alignments were coloured using Boxshade (http://www.ch.embnet.org).

Yeast two-hybrid screen

The full-length coding sequence of *PhBEH2* was amplified by reverse transcription–PCR (RT–PCR) on total RNA isolated from leaves using Phusion polymerase (Finnzymes) and primers M736 and M737. The amplification product was cloned in the *Eco*RI/*Sal*I sites in pBD-GAL4 and pAD-GAL4 (Stratagene). The PSK8-GAL4BD fusion was made by cutting *PhBEH2* yeast two-hybrid interactor 78 with *Eco*RI/*Sal*I and ligation of the fragment in pBD-GAL4. The plasmids were introduced into the yeast strain PJ69 (James et al., 1996), which carries HIS3, ADE2, and LACZ reporter genes driven by distinct GAL4-responsive promoters. For the yeast two-hybrid screen, PhBEH2/PSK8 was used as bait against a cDNA library from young petunia inflorescences (Souer et al., 2008). The library screen was performed as described previously (Quattrocchio et al., 2006). A total of 100 000 and 300 000 transformed yeast cells were screened for PhBEH2 and PSK8, respectively. Plasmid DNA was isolated from positive (HIS, ADE) colonies, transformed to *Escherichia coli*, sequenced, and subsequently reintroduced in PJ69 together with the bait plasmid expressing PhBEH2/PSK8 to confirm interactions.

For the yeast two-hybrid assay between members of the BES1/BZR1 family and GSK3/SHAGGY-like family, members from *Arabidopsis* and petunia were amplified by RT–PCR using Phusion polymerase (Finnzymes) on Petunia leaf total RNA or from existing plasmid clones. The amplification products were cloned in *Eco*RI/*Sal*I sites in pBD-GAL4 and/or pAD-GAL4 (Stratagene).

BiFC (bimolecular fluorescence complementation) analysis

For expression in protoplasts, full-length coding sequences and the N- and C-terminal halves of yellow fluorescent protein (YFP) were amplified with primers containing attB sites facilitating (Multisite) Gateway cloning (Invitrogen). After recombination in pDONR vectors, the obtained entry clones were recombined with the destination vectors V141 (pK7FWG2) to generate PhBEH2-GFP (green fluorescent protein) or V137 (pK2GW7.0) to generate the split YFP versions. The Gateway vector V154 (pB7WGY2.0) expressing free YFP was used as a control.

Accession numbers

Sequence data from this article can be found in the GenBank data library under accession numbers KC633285 (PhDWARF7), KC633286, (PhCPD), KC633287 (PhCYP85), KC633288 (PhDWARF5), KC633289 (PhDET3), KC633290 (PhSMT2), KC633291 (PhROT3), KC633292 (PhDIM), KC633293 (PhDWARF4), KC633294 (PhDET2), KC633295 (PhBRI1), KC633296 (PhPSK9), KC633297 (PhPSK8), KC633298 (PhBEH2), KC633299 (PROTEIN INTERACTING with BEH2 1 (PIB1), KC633300 (PIB8), KC633301 (PIB16), KC633302 (PIB16), KC633303 (PIB25), KC633304 (PIB41), KC633305 (PIB60), KC633306 (PIB67), KC633307 (PIB81), KC633308 [PROTEIN INTERACTING with GSK3 1 (PIG1)], KC633309 (PIG3), KC633310 PIG4), KC633311 (PIG16), KC633312 (PIG21), KC633313 (PIG28) and KC633314 (PIG93).

Results

Identification of petunia sterol/BR biosynthesis and signalling mutants

In order to study BR biosynthesis and signalling in petunia, populations of the mutagenic *Petunia hybrida* line W138 were visually screened (Gerats et al., 1990; van Houwelingen et al., 1998). Nineteen mutant families (Table 1) with similar phenotypes were identified that phenocopy sterol/BR biosynthesis or signalling mutants from other species. All mutants exhibited the reduced stature and dark round shiny leaves typical for plants with BR deficiency (Fig. 2A, B). Due to their appearance, these mutants were named *compact disc* (*cd*).

In order to identify whether these mutants are BR biosynthesis or signalling mutants, individual mutant plants were sprayed 2–3 times a week with 1 µM 24-epibrassinolide. Of 19 mutant families, 18 clearly responded to the steroid, resulting in elongation of internodes, petioles, and leaves (Table 1, Fig. 2C). Complementation of the mutant phenotype in these plants indicated that these are sterol/BR biosynthesis mutants. Only one mutant family, $cd10^{P2020}$, did not respond to exogenous application of 24-epibrassinolide, suggesting that it is mutated in a BR signalling gene.

Cross-pollination between the mutant families revealed that the 19 mutants could be assigned to five different complementation groups (see Table 1). Ten families identified at Enza Zaden harbour a *dTph1* insertion in the same BR biosynthesis gene, *CD9*. As some of these families are derived from a common ancestor (see Table 1), it is likely that they represent only three different alleles. Taken together, the total number of sterol/BR biosynthesis gene hits is four, while the remaining mutant *cd10* represents a signalling mutant. All mutants have a short robust stature with thick, small, round, and dark-green leaves, with only some slight differences in the severity of the dwarfish phenotype between the complementation groups. The *cd10* mutants remained extremely small and, even after growing for months in the greenhouse, did not flower. The biosynthesis mutants clearly grew slowly and flowered later, and fertility seemed to be reduced in the *cd2* and especially in the *cd1* mutants.

BR and steroid levels in cd mutants

To characterize the mutations further, quantitative GC-MS analysis was used to measure endogenous steroid and BR levels in the wild type and *cd* mutants (Tables 2, 3). Wild-type petunia, like tomato, does not accumulate BL, but rather castasterone (CS), as the bioactive compound (Table 2). In the *cd1* mutant, the levels of CS and 6-deoxoCS were greatly reduced. The amounts of sterols were similar to those of the wild type, indicating no lesion in sterol biosynthesis. Therefore, *cd1* seems to be mutated in an unknown brassinosteroid enzyme. Concomitantly with a large decrease in the CS and 6-deoxoCS levels, the *cd2* mutant overaccumulated 22-OH-CR, indicating a mutation in the petunia *CYP90A1* (*CPD*) gene. In the *cd3* mutant, massive accumulation of 6-deoxoCS was observed, suggesting that this mutant has a defect in the conversion of 6-deoxoCS to castasterone, a reaction that in tomato is catalysed by the CYP85A1 enzyme (Bishop et al., 1999). In the *cd9* mutant, sterols are abundant but the pathway end-products CS and 6-deoxoCS were severely reduced. In *cd9*, 22-OH-CR, 22-OH-3-one, and 6-deoxoCT accumulated, suggesting a mutation in a CYP90C1- or CYP90D1-like

Fig. 2. Petunia BR biosynthesis and signalling mutants. (A) Phenotype of 4-week-old *Petunia hybrida* wild-type (left) and *cd1*^{W503}, *cd2*^{P2015}, *cd3*^{D2213}, and *cd9*^{P2023} plants. (B) Top view of a *cd1*^{W503} mutant. (C) Complementation of the *cd2*^{P2013} mutant but not of the *cd10*^{P2020} mutant by spraying with 1 μM 24-epibrassinolide (+BL). Mock indicates plantlets treated with a solution without 24-epibrassinolide.

Table 1. Overview of cd alleles and results of complementation experiment by brassinolide treatment.

Locus	Allele	Origin	Complemented by BR[a]
cd1	cd1-W503	VU	Yes
	cd1-K2078	Nijmegen	Yes
cd2[b]	cd2-W2254	VU	Yes
	cd2-P2013	ENZA	Yes
	cd2-P2015	ENZA	Yes
	cd2-P2018	ENZA	Yes
cd3	cd3-D2213	VU	Yes
	cd3-P2014	ENZA	Yes
cd9[c]	cd9-P2016	ENZA	Yes
	cd9-P2019	ENZA	Yes
	cd9-P2021	ENZA	Yes
	cd9-P2022	ENZA	Yes
	cd9-P2023	ENZA	Yes
	cd9-P2024	ENZA	Yes
	cd9-P2025	ENZA	Yes
	cd9-P2026	ENZA	Yes
	cd9-P2027	ENZA	Yes
	cd9-P2028	ENZA	Yes
cd10	cd10-P2020	ENZA	No

[a] Complementation was tested by spraying with 1 μM 24-epibrassinolide.
[b] Due to reduced fertility, *cd2* allelism was confirmed molecularly as independent insertions in *PhCYP90A1* (see text).
[c] *cd9*^{P2016}, *cd9*^{P2019}, *cd9*^{P2022}, *cd9*^{P2024}, *cd9*^{P2025}, *cd9*^{P2026}, *cd9*^{P2027}, and *cd9*^{P2028} are derived from a common ancestor and thus might represent identical alleles.

Table 2. Endogenous BR levels of wild-type petunia (W138), cd1, cd2, cd3, and cd9 plants (ng kg^{-1} FW).

	Wild type	*cd1*	*cd2*	*cd3*	*cd9*
Tissue FW (g)	95.0	88.0	82.1	102.0	26.6
BR/steroid					
BL	ND	ND	ND	ND	ND
CS	943	24	92	15	63
6-DeoxoCS	7621	157	86	59 668	84
6-DeoxoTY	566	610	488	1008	512
6-Deoxo3DT	116	135	202	230	257
6-DeoxoTE	191	142	170	109	ND
6-DeoxoCT	4471	7770	4055	1668	12 260
TY	Lost	Lost	Lost	Lost	ND
3DT	ND	ND	ND	ND	ND
TE	ND	ND	ND	ND	ND
22-OH-CR[a]	754	1151	4337	392	4956
22-OH-3-one[a]	2858	5275	1208	1471	16 739
3-Epi-6-deoxoCT[a]	6571	3891	1531	5715	10 777

FW, fresh weight; ND, not detectable; lost, internal standards not recovered.
[a] Quantified by peak areas because internal standards are unavailable.
Abbreviations of BRs: BL (brassinolide), CS (castasterone), 6-deoxoCS (6-deoxocastasterone), 6-deoxoTY (6-deoxotyphasterol), 6-deoxoTE (6-deoxoteasterone), 6-deoxoCT (6-deoxocathasterone), TY (typhasterol), 3DT (3-dehydroteasterone), TE (teasterone), 22-OH-CR [(22*S*)22-hydroxycampsterol], 22-OH-3-one [(22*S*,24*R*)-22-hydroxy-5-ergostan-3-one], 3-epi-6-deoxoCT (3-epi-6-deoxocathasterone).

Table 3. Endogenous steroid levels of wild-type petunia (W138), cd1, and cd9 plants (g kg^{-1} FW).

Steroid	Wild type	*cd1*	*cd9*
Cholesterol	3.4	4.7	4.5
24-Methylenecholesterol	24.0	19.0	30.0
Campesterol	30.2	42.0	71.5
Isofucosterol	140.2	392.0	376.0
Stigmasterol	19.3	14.0	31.9
Sitosterol	36.0	79.9	92.3

enzyme. In conclusion, it was found that *cd1*, *cd2*, *cd3*, and *cd9* are all BR deficient.

cd2 and cd3 encode the petunia CYP90A1 and CYP85A1 homologues, respectively

The biochemical analysis of BR content in *cd* mutants in some cases provided clues on the identity of the affected gene. Using a combination of database mining and primer design based on sequences of Solanaceae species (e.g. tomato, tobacco, potato, etc.; Supplementary Table S1 at *JXB* online), it was possible to clone gene and/or cDNA fragments of petunia homologues of the *Arabidopsis* BR biosynthesis genes *CYP90A1*, *DWF5*, *CYP85A1*, *DIM*, *DWF7*, *SMT2*, *DETIOLATED2* (*DET2*), *DWARF4* (*DWF4*), and *CYP90C1* (*ROT3*), and a number of signalling genes (see below).

The obtained sequence data of petunia BR biosynthesis genes were used to determine in each *cd* mutant if any of these known genes is disrupted by a *dTph1* insertion. When DNA isolated from *cd2* plants (Fig. 3A) was used as a template, PCRs identified *dTph1* insertions at different sites in the petunia homologue of the BR biosynthesis gene *CYP90A1* (Szekeres et al., 1996) (Fig. 3D, F). The *dTph1* transposon insertion in allele $cd2^{W2254}$ was found in the first exon, ~80 nucleotides after the ATG. The other three mutants ($cd2^{P2013}$, $cd2^{P2015}$, and $cd2^{P2018}$) carry a *dTph1* insertion at different sites in the second exon (Fig. 3D). Plants in progeny of $cd2^{W2254}$ occasionally gave rise to revertant shoots (Fig. 3B).

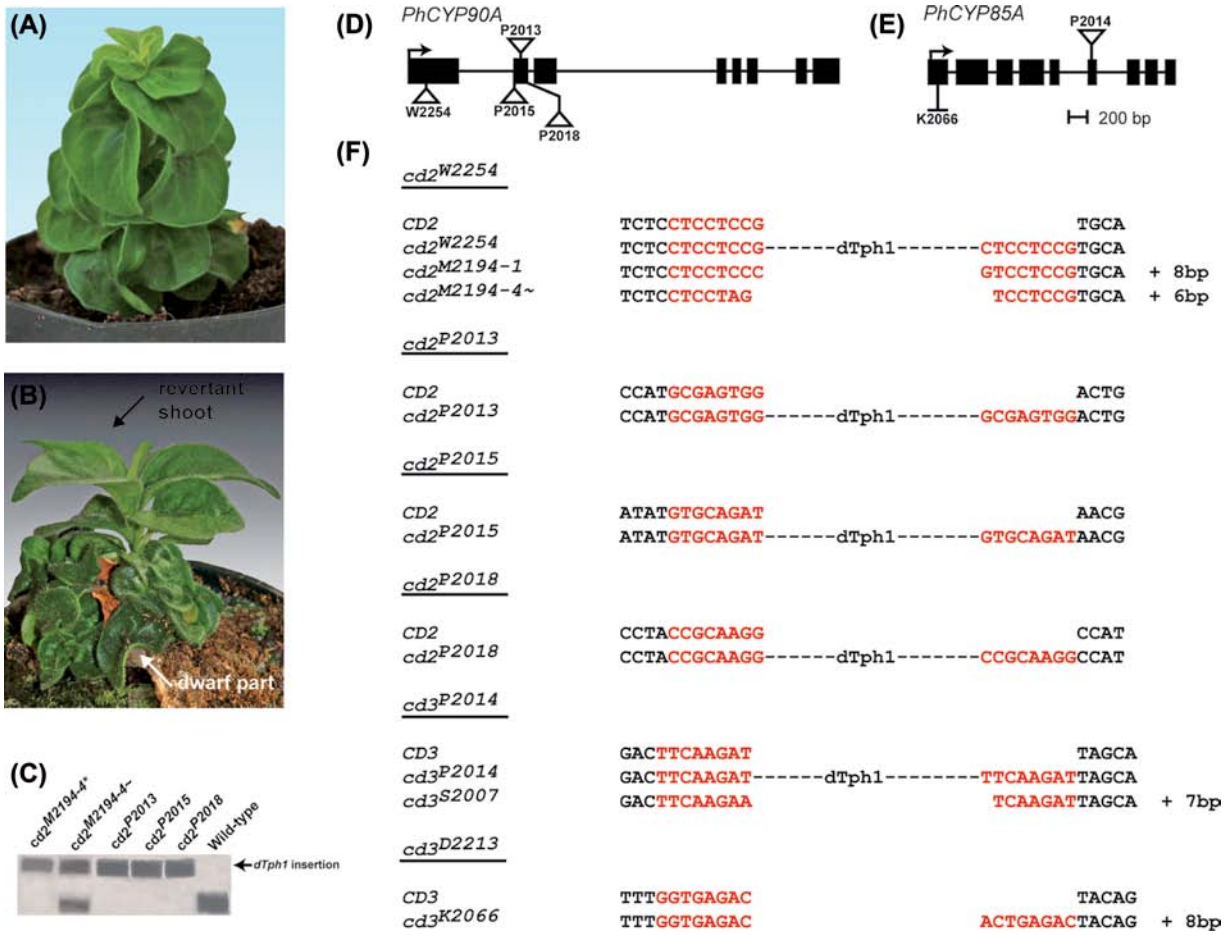


Fig. 3. Molecular analysis of two BR biosynthesis mutant families. (A) Mutant phenotype of a $cd2^{W2254}$ plant. (B) A $cd2^{W2254}$ plant that develops a revertant shoot ($cd2^{M2194-4}$). (C) PCR with *CYP90A1*-specific primers on genomic DNA extracted from plants carrying various *CYP90A1* alleles. (D) Schematic drawing of the petunia *CYP90A1* gene and mutant alleles. (E) Schematic drawing of the petunia *CYP85A1* gene and mutant alleles. In D and E, boxes represent exons and the thin lines represent introns. The triangles and line illustrate the *dTph1* transposon insertions and footprint in the indicated alleles. (F) Sequence analysis of the *dTph1* insertions in various *CYP90A1* and *CYP85A1* alleles. The red sequence indicates the target site duplication.

PCR and sequence analysis on genomic DNA extracted from a revertant shoot ($cd2^{M2194\text{-}4}$) revealed excision of *dTph1* from *CYP90A1*, leaving a 6 bp footprint (Fig. 3F). Furthermore, a stable *cd2* mutant ($cd2^{M2194\text{-}1}$) was identified in which excision of *dTph1* created an 8 bp footprint (Fig. 3F).

The identity of $cd3^{P2014}$ was revealed by mining a 3D indexed petunia insertion database generated from the ENZA population (Vandenbussche et al., 2008). A transposon-flanking sequence unique for family P2014 showed homology to *CYP85A1* (Bishop et al., 1999). The *dTph1* element was identified in exon 6 of the petunia *CYP85A1* homologue (Fig. 3E). In progeny of $cd3^{P2014}$, a stable mutant was shown to carry a mutant allele containing a 7 bp footprint ($cd3^{S2007}$). In plants carrying the $cd3^{D2213}$ allele, a transposable element could not be identified in the petunia *CYP85A1* homologue. However, sequencing of the *CYP85A1* coding sequence from one of the progeny (family *cd3-K2066*) showed an 8 bp footprint in exon 1, indicating the previous existence of a transposable element at this site (Fig. 3E, F).

Analysis of *cd1* and *cd9* mutants did not reveal transposon insertions in the putative petunia homologues of *DWARF5*, *DET3*, *DIM*, *DWARF7*, *SMT2*, *DET2*, *DWARF4*, or *ROT3*. It must be said that the complete sequences of these genes are currently unknown. As 3' sequences (*DIM*), 5' sequences (*DWARF5*, *CYP85*, *DWARF7*, *SMT2*), or both (*DET3*, *DET2*, *DWARF4*, *ROT3*) are unknown, it cannot be excluded that there are *dTph1* insertions present in these parts of the gene. In the 3D indexed petunia insertion database (Vandenbussche et al., 2008), transposon insertions were identified in petunia homologues of DIM and DET2, both without phenotypic consequences, suggesting that these genes are redundant in petunia (see the Supplementary data at *JXB* online).

cd10 is mutated in the petunia BRI homologue

In *Arabidopsis*, BRs are perceived by the leucine-rich repeat (LRR) receptor-like kinase BRI1 (Li and Chory, 1997). From the *P. hybrida* expressed sequence tag (EST) collection, a sequence that showed a high degree of similarity to the 3' end of *BRI1* (GenBank accession no. CV294320) was retrieved. The full-length mRNA sequence of W138 *PhBRI1* was obtained via RACE-PCR and SOTI-PCR (see the Materials and methods). Comparison of the derived amino acid sequence of PhBRI1 with AtBRI1, OsBRI1, LeBRI1, and NbBRI1 revealed 63.3, 53.4, 84.1, and 91.3% identity, respectively (Fig. 4A; Supplementary Fig. S1 at *JXB* online). Similar to the other identified BRIs, PhBRI1 has an LRR domain, a putative leucine-zipper motif, a transmembrane domain to anchor the protein in the plasma membrane, and a cytoplasmic kinase domain (Fig. 4B; Supplementary Fig. S1). The BRI1 extracellular domain of petunia contains 25 tandem N-terminal LRRs, which is interrupted by a 68 amino acid non-repetitive island between the 21st and 22nd LRR.

Because $cd10^{P2020}$ failed to respond to application of 24-epibrassinolide, *cd10* was assumed to be mutated in a BR signalling gene (Fig. 2C). One of the prime candidates for being hit in $cd10^{P2020}$ is the homologue of the *Arabidopsis* BR receptor, *BRI1*. PCR analysis on genomic DNA with *PhBRI1*-specific primers revealed that *cd10* plants indeed contain an insertion in the 5' part of the gene (Fig. 4C, D). Sequence analysis of the 5' part of *PhBRI1* in *cd10* revealed that the *dTph1*

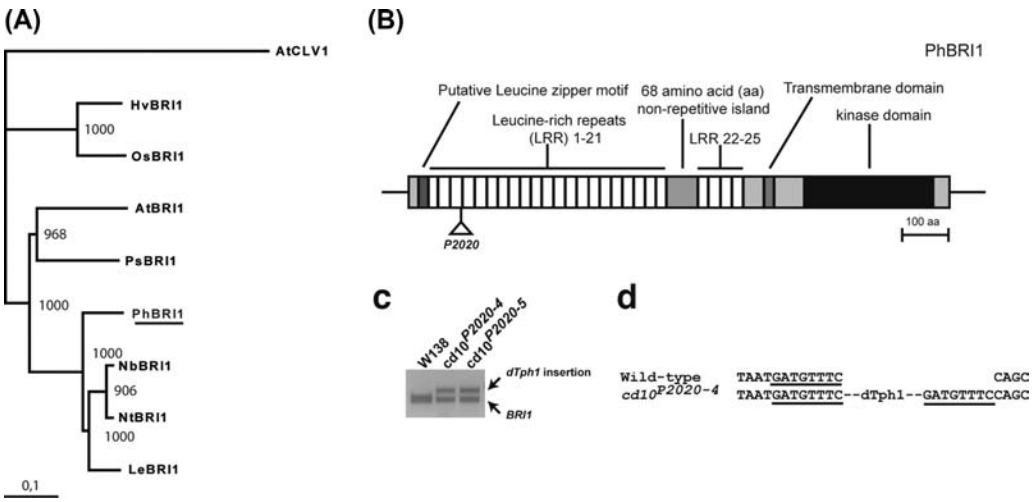


Fig. 4. Characterization of the BRI1 receptor from petunia. (A) Phylogenetic tree constructed using derived amino acid sequences of the BRI1 homologue isolated from petunia and various other species [At, *Arabiopsis thaliana*; Hv, *Hordeum vulgare*; Os, *Oryzia sativa*; Le, *Lycoperscicon esculentum* (*Solanum lycopersicum*); Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Ps, *Pisum sativum*; and Ph, *Petunia hybrida*]. The petunia *BRI1* protein (*PhBRI1*) is underlined. *CLAVATA1* from *Arabidopsis* was used as an outgroup. GenBank accession numbers are provided in Supplementary Table S2 at *JXB* online. (B) Schematic drawing of PhBRI1 with its predicted functional domains. The triangle indicates the *dTph1* transposon insertion in $cd10^{P2020}$. (C) PCR with *BRI1*-specific primers on genomic DNA extracted from $cd10^{P2020}$ and wild-type plants. (D) Sequence analysis of the *dTph1* insertion in *cd10*. The underlined sequence indicates the target site duplication.

transposable element was inserted in the third LRR sequence motif (Fig. 4B).

Identification of a BEH2 orthologue in petunia

To explore the BR signalling cascade in petunia further, the aim was to identify signalling components downstream of PhBRI1. In *Arabidopsis*, the *BES1/BZR1* transcription factor family plays an important role in the regulation of BR-responsive genes (Zhao et al., 2002). A PCR-based approach was performed to identify potential petunia homologues. Primers were developed based on the sequences of all six BES1/BZR1 family members of *Arabidopsis* and a nested 3' RACE-PCR on petunia leaf cDNA yielded a PCR fragment of the desired size. Sequence analysis revealed high sequence identity with members of the BES1/BZR1 family. The 5' end was isolated by SOTI-PCR (see the Materials and methods). The derived amino acid sequence has the highest sequence identity to the BES1/BZR1 family member AtBEH2 (64.2%) and therefore the clone was named *PhBEH2* (Fig. 5A; Supplementary Fig. S2 at *JXB* online). Like the previously characterized BES1/BZR1 family members from *Arabidopsis*, *PhBEH2* has a putative bipartite nuclear localization signal (NLS) at its N-terminus. The central part of PhBEH2 is rich in serine/threonine residues that match with the phosphorylation sites for GSK3 kinases (Wang et al., 2002). In addition a PEST motif was identified that seems to play an important role in protein degradation as BES1 and BZR1 proteins were stabilized in *bes1-D* and *bzr1-D* mutants carrying a mutation in this motif (Wang et al., 2002; Yin et al., 2002).

PhBEH2 binds to a GSK3/SHAGGY-like kinase and 14-3-3 proteins

To identify proteins that interact with PhBEH2, a yeast two-hybrid screen was performed. As many transcription factors carry activation domains, auto-activation of the full-length cDNA of *PhBEH2* on yeast reporters was tested first. Indeed, introduction of the PhBEH2–GAL4-binding domain fusion resulted in some activation of the HIS reporter, but selection on histidine and adenine resulted in no growth (Supplementary Fig. S4 at *JXB* online) Subsequently, the full-length cDNA of *PhBEH2* was used as bait to screen a two-hybrid cDNA library made from young petunia inflorescence, and 12 interacting proteins were found (Fig. 5B, C). Sequence analysis showed that five of these proteins were 14-3-3 proteins. Another interesting interacting partner, which was named PSK8, had high similarity to the GSK3-like kinase BIN2 from *Arabidopsis* (Fig. 5C). Other interaction partners include proteins homologous to SECRET AGENT (SEC), which is an O-linked N-acetylglucosamine transferase (OGT), ARIA (ARMADILLO Repeat protein Interacting with ABF2), TPL (TOPLESS), and two RRM (RNA recognition motif) proteins of which one is a homologue of FPA (Flowering Protein A; Fig. 5C).

To verify some of the interactions found in yeast in plant cells, BiFC was used in petunia leaf protoplasts (Hu et al., 2002). As numerous interactions with PhBEH2 were detected, the search was restricted to interactions known from *Arabidopsis* research. In addition, the aim was to verify the interaction with the potential OGT PhSEC. As positive controls, free YFP as well as a fusion between PhBEH2 and GFP were used (Fig. 5D, E). When fused to GFP, BEH2 was mainly detected in the nucleus, but clearly in the cytoplasm too. For BiFC, translational fusions of *PhBEH2*, *Ph14-3-3k*, *PSK8*, and *PhSEC* with the N- and C-terminal halves of *YFP* were generated. Interaction was detected between PhBEH2 and 14-3-3k (Fig. 5G), PSK8 (Fig. 5F, H), and PhSEC (Fig. 5I). The interaction between PhBEH2 and 14-3-3k occurred in the cytoplasm and nucleus, whereas interaction with PSK8 and PhSEC appeared almost exclusively in the nucleus. It thus seems that at least in the case of 14-3-3k, PSK8, and PhSEC, the interaction with PhBEH2 can also occur in plant cells.

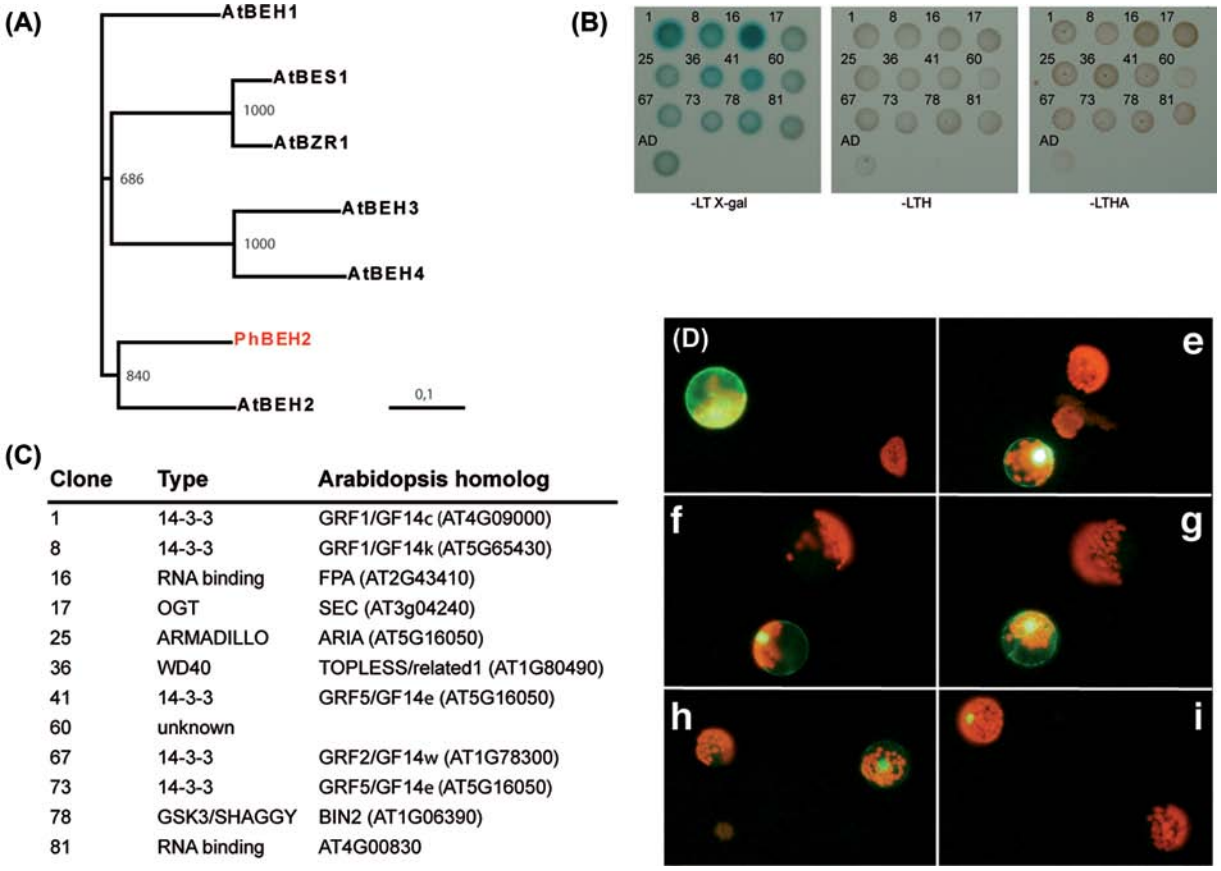
Isolation and characterization of two group II members of the GSK3/SHAGGY-like family from petunia

One of the interacting partners of PhBEH2 was PSK8, a protein that has strong homology to the GSK3-like kinase BIN2 from *Arabidopsis* (Fig. 6A; Supplementary Fig. S3 at *JXB* online). From a previous yeast two-hybrid screen, performed in the authors' laboratory, using one of the transcription factors involved in flower pigmentation as bait, another GSK3-like kinase was identified as an artefact. As this clone lacked the 5' half of the cDNA, SOTI-PCRs (see the Materials and methods) were performed to isolate the remaining part. This clone was renamed *PSK9*. Both *PSK8* and *PSK9* are related to BIN2 as they group together with the other group II ASKs (Fig. 6A). Kim et al. (2009) showed that the phosphatase BSU1 inactivates BIN2 by dephosphorylation at pTyr200. This tyrosine residue is also conserved in PSK8 and PSK9 (Supplementary Fig. S3).

To gain more insight into the function of PSK8, a yeast two-hybrid screen was performed, where full-length PSK8 was used as bait to screen a cDNA library made from young petunia inflorescences. Eight different cDNAs that interacted with PSK8 were identified. The interacting partners included two different LRR-extensins, REMORIN, and several unknown proteins. (Fig. 6B, C).

Key regulators of the petunia/Arabidopsis BR pathway are functionally homologous

PSK8 and *PSK9* are highly homologous to the group II GSK3/SHAGGY-like kinases from *Arabidopsis*, and this prompted the examination of whether they also have functional homology. In order to investigate this, yeast two-hybrid assays were performed with members of the BES1/BZR1 family and the group II GSK3/SHAGGY-like kinases from *Arabidopsis* and petunia. AtBIN2 interacted with AtBES1, AtBZR1, and also with BEH2 from petunia (Fig. 7). A similar behaviour was found for the petunia BIN2 homologue PSK8. However, PSK9 did not bind to PhBEH2 or to the *Arabidopsis* members. As the interaction with PSK8/



(C)

Clone	Type	Arabidopsis homolog
1	14-3-3	GRF1/GF14c (AT4G09000)
8	14-3-3	GRF1/GF14k (AT5G65430)
16	RNA binding	FPA (AT2G43410)
17	OGT	SEC (AT3g04240)
25	ARMADILLO	ARIA (AT5G16050)
36	WD40	TOPLESS/related1 (AT1G80490)
41	14-3-3	GRF5/GF14e (AT5G16050)
60	unknown	
67	14-3-3	GRF2/GF14w (AT1G78300)
73	14-3-3	GRF5/GF14e (AT5G16050)
78	GSK3/SHAGGY	BIN2 (AT1G06390)
81	RNA binding	AT4G00830

Fig. 5. Characterization of PhBEH2 and the identification of binding partners by a yeast two-hybrid screen. (A) Phylogenetic tree constructed using derived amino acid sequences of PhBEH2 and the BES1/BZR1 family from *Arabidopsis*. GenBank accession numbers are provided in Supplementary Table S2 at *JXB* online. (B) The full-length sequence of *PhBEH2* was fused to the yeast GAL4-binding domain and screened against a cDNA library made from young petunia inflorescences. Interactions were measured by growth on medium lacking histidine (–LTH) or histidine and adenine (–LTHA), and by blue colouring using 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (–LT X-gal). (C) Classification of the positive clones from B. As indicated, for some proteins, a homologue with known function is found in *Arabidopsis*. (D–I) Verification of interactions in petunia protoplasts by BiFC (Hu et al., 2002). (D) Free YFP; (E) PhBEH2–GFP; (F) PhBEH2–YFPN and PSK8–YFPC; (G) PhBEH2–YFPN and 14-3-3κ–YFPC; (H) PSK8–YFPN and PhBEH2–YFPC; (I) PhSEC–YFPN and PhBEH2–YFPC. All constructs are driven by the 35S promoter. All panels show a transformed as well as an untransformed protoplast.

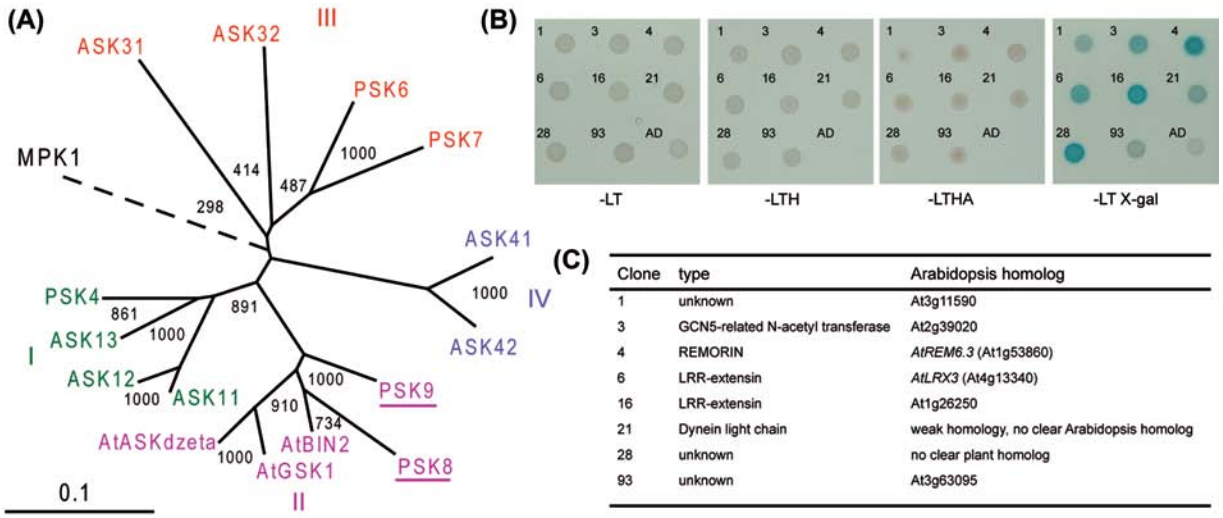
BIN2 showed that the fusions of BES/BZR proteins with GAL4-AD are functional in yeast, it seems that either PSK9 does not interact with the BES1/BZR1 family or the fusion between PSK9 and the GAL4-binding domain is somehow not functional. The yeast-two hybrid results suggest that group II GSK3/SHAGGY-like kinases and members of the BES/BZR1 family from *Arabidopsis* and petunia are structurally highly homologous.

Discussion

The synthesis of BRs and the signalling cascade downstream of the bioactive compounds have been largely elucidated over the past 10 years (Fujioka and Yokota, 2003; Clouse, 2011). As in many plant research areas, these findings basically originate from *Arabidopsis*. Partial confirmation of evolutionary conservation as well as additions came from numerous reports on BR biosynthesis and signalling in other species (e.g. Nomura et al., 2001; Montoya et al., 2002; Bai et al., 2007; Koh et al., 2007). The available resources in petunia have been used to identify large parts of the BR biosynthesis and signalling route. The initial results point to conserved BR biosynthesis genes and interacting signalling proteins, as well as to potential new relationships between hormone signalling pathways.

Novel and known BR mutants

Generally, a mutation in a BR biosynthesis gene causes the accumulation of a precursor(s) that should have been metabolized and a reduction of the (end)product(s). In the *cd2* mutant, accumulation of 22-OH-CR, which has been recognized as the first metabolite of campesterol in the early C-22 oxidation branch of BR biosynthesis, was observed (Fujita et al., 2006; Fig. 1). A high level of accumulation of 22-OH-CR was also observed by Ohnishi et al. (2012) when



Clone	type	Arabidopsis homolog
1	unknown	At3g11590
3	GCN5-related N-acetyl transferase	At2g39020
4	REMORIN	*AtREM6.3* (At1g53860)
6	LRR-extensin	*AtLRX3* (At4g13340)
16	LRR-extensin	At1g26250
21	Dynein light chain	weak homology, no clear Arabidopsis homolog
28	unknown	no clear plant homolog
93	unknown	At3g63095

Fig. 6. Phylogenetic analysis of PSK8/9 and the identification of PSK8 binding partners by a yeast two-hybrid screen. (A) Phylogenetic tree constructed of derived amino acid sequences of PSK8/9 and GSK3-like kinases from *Arabidopsis* and petunia. MPK1 from *Arabidopsis* was used as an outgroup to construct the tree, but is not shown in this tree. GenBank accession numbers are shown in Supplementary Table S2 at *JXB* online. (B) Interaction of PSK8 with eight different clones in a yeast two-hybrid assay. Interactions were measured by growth on medium lacking histidine (–LTH) or histidine and adenine (–LTHA), and by blue colouring using 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (–LT X-gal). (C) Classification of the positive clones from B. When a clear homologue in *Arabidopsis* is found, their AGI identifier is listed.

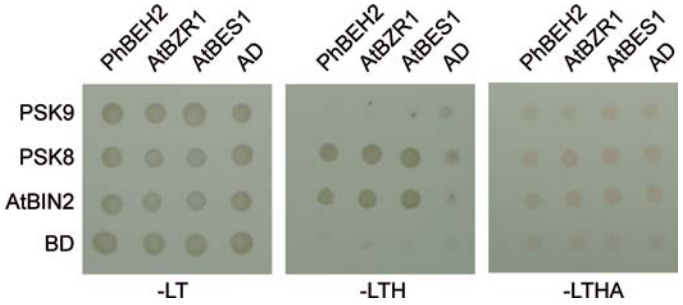


Fig. 7. The BES1/BZR1 family and group II GSK3/SHAGGY-like kinases from petunia and *Arabidopsis* are functionally homologous. Full-length sequences of AtBIN2, PSK8, and PSK9 were fused to the GAL4-binding domain, and AtBZR1, AtBES1, and PhBEH2 to the GAL4 activation domain, and tested for interaction. Interactions were measured by growth on medium lacking histidine (–LTH) or histidine and adenine (–LTHA).

they researched the *cpd*/*cyp90a1* mutant. The biochemical evidence strongly suggested that *CD2* is homologous to *CPD*/*CYP90A1*, although the product, 22-OH-4-en-3-one, has not been analysed. Indeed, molecular analysis confirmed *CD2* as the petunia homologue of *CYP90A1* (Fig. 3; Szekeres et al., 1996). In *cd3*, the build-up of 6-deoxoCS and the concomitant reduction of CS was observed, indicating a lesion in *CYP85A1*. Once more the biochemical data were confirmed as *cd3* was found to be mutated in the petunia homologue of *CYP85A1* (Fig. 3; Bishop et al., 1996).

Endogenous levels of upstream BRs in *cd1* are quite similar to those of the wild type, and sterol levels are normal. However, the levels of both 6-deoxoCS and CS, which appear in the last steps of biosynthesis, are reduced. No accumulation of other measured BR biosynthesis intermediates could be identified. Thus, the nature of the *cd1* mutation is currently unknown and it might represent an enzyme that has not been identified yet in any other plant species. In *cd9*, the levels of 22-OH-3-one, 3-epi-6-deoxoCT, and 6-deoxoCT that are substrates of CYP90C1 (ROT3)/CYP90D1 were higher as compared with the wild type, indicating a potential deficiency in a CYP90C1/CYP90D1-like activity (Fig. 1). However, the *Arabidopsis* CYP90C1/CYP90D1 double mutant does not accumulate 22-OH-3-one, 3-epi-6-deoxoCT, and 6-deoxoCT (Ohnishi et al., 2006). It thus seems that, in contrast to the situation in *Arabidopsis*, the CYP90C1 (ROT3)/CYP90D1 activity in petunia is encoded by a single enzyme. *dTph1* insertions could not be found in the *ROT3* homologue of *cd9*, but it cannot be ruled out that the insertion might be located in the 5' or 3' part that currently is lacking. Altogether, the evidence available points to unknown BR biosynthesis enzymes or perhaps regulators of BR biosynthesis enzymes mutated in *cd1* or *cd9*. Cloning of the responsible genes can be accomplished in the future by using amplified fragment length polymorphim (AFLP)-based transposon display or high throughput sequencing of *dTph1*-flanking sequences (Van den Broeck et al., 1998; Vandenbussche et al., 2008).

BR signalling mutants

The single signalling mutant that was identified, *cd10*, was shown to harbour an insertion in the petunia homologue of the BR receptor, *BRI1* (Li and Chory *et al.*, 1997). The *cd10* mutant exhibits an extremely dwarfed stature and did not flower at all. All known *bri1* mutants do flower, except the

osbri1 mutant from rice (Nakamura et al., 2006). The strong mutants from rice and petunia might reflect complete loss of BRI1 function, whereas in other species either only partial loss-of-function alleles were identified, partial redundancy in BRI1 function might exist, and/or another non BR-dependent flowering pathway plays a dominant role.

In an amino acid alignment of BRI1 from different species, the highest similarity was observed in the crucial kinase domain (Supplementary Fig. S1 at *JXB* online; Tang et al., 2008; X. Wang et al., 2008). The extracellular domain of PhBRI1 contains 25 tandem N-terminal LRRs and a 68 amino acid non-repetitive island between the 21st and 22nd LRR. This island together with LRR22 has proved to be important for BR binding by BRI1 in *Arabidopsis* (Kinoshita et al., 2005). Thus it seems that the perception of the BR hormone is evolutionarily well conserved.

Apart from PhBRI1, petunia homologues belonging to the BES1/BZR1 family as well as GSK3-like kinases, key components of the BR signalling pathway in *Arabidopsis*, were cloned. The BES1/BZR1 family homologue that was isolated from petunia (*PhBEH2*) showed the highest sequence identity to the BES1/BZR1 family member BEH2 (64.2%). In *Arabidopsis*, the *bzr1/bes1* double mutant only exhibits a semi-dwarf phenotype. It thus seems likely that BEH2 and perhaps other members of the BES1/BZR1 family are redundant to BES1 and BZR1.

Interactions between BR signalling components

The interaction spectrum of PhBEH2 in yeast supports a role in BR signalling. First, PhBEH2 is capable of interacting with *Arabidopsis* BIN2 (Fig. 7), a proven negative regulator of BR signalling (Li et al., 2001). Secondly, the PhBEH2 protein interacted with several 14-3-3 proteins (Fig. 5C). Previous research in *Arabidopsis* showed that 14-3-3 proteins increase the cytoplasmic retention of phosphorylated BES1/BZR1 (Gampala et al., 2007). Mutation of a BIN2 phosphorylation site in BZR1 was shown to abolish 14-3-3 binding, leading to increased nuclear localization of the BES1/BZR1 protein (Bai et al., 2007; Gampala et al., 2007). Like AtBZR1 and AtBES1, PhBEH2 also possesses the conserved putative 14-3-3-binding site, suggesting that the regulation of the BZR1/BES1 family by 14-3-3 proteins might be conserved among plants. Thirdly, PhBEH2 interacted with a petunia GSK3-like kinase (PSK8) that belongs to the group II GSK3-like kinases (Figs 5, 7). *Arabidopsis* group II GSK3-like kinases have been shown to regulate BR signalling negatively by phosphorylating the BZR1/BES1 family (Vert and Chory, 2006; Yan et al., 2009; Rhozon *et al.*, 2010). To reveal the involvement of PSK8 in BR signalling, phosphorylation of PhBEH2 by PSK8 should be demonstrated and knock-outs might ultimately reveal their function. In addition to PSK8, another group II member (PSK9) was also identified in petunia. Based solely on similarity, it seems likely that PSK9 is also involved in BR signalling. The lack of interaction with PhBEH2 and *Arabidopsis* BES1 and BZR1 might be due to aberrant processing of the PSK9–GAL4 fusion RNA and/or protein in yeast.

Identification of new interactions

The present yeast two-hybrid screen with PhBEH2 revealed several interacting proteins that function in diverse signal transduction pathways. This suggests that PhBEH2 could be an important hub between BR and other signalling pathways. Recently, a number of reports highlighted interactions between BR signalling and other hormone pathways (reviewed by Choudhary et al., 2012).

One of the most interesting partners of PhBEH2 is a protein homologous to the *Arabidopsis* gene *SECRET AGENT* (*SEC*) encoding an OGT (Hartweck et al., 2002, 2006). For SEC, the interaction was confirmed in yeast using BiFC in petunia protoplasts (Fig. 5I). SEC catalyses the transfer of a single O-linked-N-acetylglucosamine to specific serine/threonine residues. SEC is partly redundant with another OGT in *Arabidopsis* called SPINDLY (SPY). Shimada et al. (2006) reported that mutation of the rice SPINDLY gene (OsSPY) reduced the expression of several BR biosynthesis genes and *OsBRI1*. As expected, the levels of sterol and BR compounds were slightly up-regulated in the *OsSPY* knock-out mutant.

O-GlcNAcylation of target proteins might affect their activity, localization, stability, and interactions with other proteins. The fact that OGTs add acetylglucosamine to serine/threonine residues of proteins suggests a model where OGTs and GSKs compete for modification of these residues in the BES/BZR family of proteins. Such a scenario, where an OGT competes with a kinase, is a widely recognized mechanism in animal research (Kamemura et al., 2002; Z. Wang et al., 2008; Butkinaree et al., 2009). Most strikingly, there are specific examples where GSK3 phosphorylation and *O*-GlcNAcylation occur on the same site of proteins (Wang et al., 2007). It would be interesting to see whether PhSEC adds acetylglucosamine to PhBEH2 and, if so, what the consequences are for the function of PhBEH2.

Multiple links between petunia BR signalling components and other pathways were identified. For instance, PhBEH2 interacts with a protein that has high sequence homology to ARIA, a positive regulator of the ABA signalling cascade. PhBEH2 also interacts with two proteins containing RRMs. One of the RRM proteins is a homologue of *FPA*, a gene that regulates flowering time in *Arabidopsis* via a pathway that is independent of daylength (Schomburg et al., 2001). Various connections between flowering and BRs have been revealed (Domagalska et al., 2007; Lee et al., 2008a, *b*; Yu et al., 2008). This is consistent with the finding that BR biosynthesis mutants as well as signalling mutants are late flowering or, like the *cd10* (petunia *bri1*) mutant, non-flowering. Although interesting, further research should confirm these findings.

Remarkably, no known homologues of the BR signalling pathway were found in the yeast two-hybrid screen performed with PSK8. Using PhBEH2 as bait, PSK8 was isolated, but using the same cDNA library the reverse interaction was not identified. It might be that none of the BES1/BZR1 family members present in the library is in-frame with the GAL4-binding domain. Nonetheless, the yeast two-hybrid screen with PSK8 might have revealed other roles of group II GSK kinases that have not been recognized yet.

PSK8 interacted with a number of proteins that are poorly characterized. Among these are a member of the *LLR-extensin* (*LRX*) and of the REMORIN family. So far, the action of GSK kinases in plants was found to occur via phosphorylation of transcription factors such as those belonging to the BES1/BZR family. Here, the identified interactions infer a direct role for GSK3-like proteins in the regulation of enzyme-like proteins, a mechanism that has interfaces with some of the roles of GSK3 in animals.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Alignment of the deduced amino acid sequences of BRI1 and homologues.

Figure S2. Alignment of the deduced amino acid sequences of BES/BZR and homologues.

Figure S3. Alignment of the deduced amino acid sequences of class II GSK3/SHAGGY-like kinases from *Arabidopsis* and petunia.

Figure S4. Yeast one-hybrid to test for autoactivation of baits.

Table S1. Primers used for PCR.

Table S2. GenBank accession numbers

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